AD_____

Award Number: W81XWH-08-1-0759

TITLE: Detachment-Induced Autophagy and Breast Cancer Cell Survival

PRINCIPAL INVESTIGATOR: Rebecca Lock

CONTRACTING ORGANIZATION: The University of California
San Francisco, CA 94143

REPORT DATE: October 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-10-2011	Final	1 Oct 2008 - 30 Sep 2011

4. TITLE AND SUBTITLE

Detachment-Induced Autophagy and Breast Cancer Cell Survival

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0759

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Rebecca Lock

E-Mail: Rebecca.Lock@ucsf.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of California
San Francisco, CA 94143

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Abstract on next page.

15. SUBJECT TERMS
autophagy, oncogenes, extracellular matrix detachment, 3D morphogenesis, cell transformation

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	13	**19b. TELEPHONE NUMBER** *(include area code)*

14. ABSTRACT

Subject: During breast cancer progression and metastasis, tumor cells acquire the ability to survive and grow in stressful microenvironments. The genetic lesions that drive proliferation and prevent cell death during tumor development are well understood, however, less is known about the contributions of pathways that allow cells to cope with environmental stress, such as autophagy. **Purpose:** Although autophagy is known to aid in cell survival in response to a wide range of stress stimuli, it remains unclear whether autophagy enhances or suppresses the development and progression of breast cancer (Mizushima et al., 2008). Understanding the role of autophagy during stress in the context of breast cancer cells will allow us to determine if autophagy could be a valuable drug target for breast cancer treatment. **Scope:** The goals of this research project are to: 1) Determine if breast epithelial cells (MCF10A cells) expressing oncogenes mutated in breast cancer initiate autophagy during extracellular matrix detachment, 2) Determine if autophagy inhibition promotes cell death during extracellular matrix detachment, alters 3D morphogenesis, and contributes to oncogenic transformation, and 3) Determine if autophagy suppression increases cell death and alters transformation in established breast cancer cell lines. Over the entire funding period of this award we have found that autophagy is induced in oncogene expressing breast epithelial cells and in two breast cancer cells lines in response to extracellular matrix detachemnet. Importantly we have found that autophagy is critical for the fitness of these cells as autophagy inhibition decreases the transformation potential of oncogene expressing breast epithelial cells and a breast cancer cell line, MDA-MB-231. Finally, we have found that autophagy

Table of Contents

FINAL REPORT: BC083204. Detachment-Induced Autophagy and Breast Cancer Cell Survival

INTRODUCTION: **Subject:** During breast cancer progression and metastasis, tumor cells acquire the ability to survive and grow in stressful microenvironments. The genetic lesions that drive proliferation and prevent cell death during tumor development are well understood, however, less is known about the contributions of pathways that allow cells to cope with environmental stress, such as autophagy. **Purpose:** Although autophagy is known to aid in cell survival in response to a wide range of stress stimuli, it remains unclear whether autophagy enhances or suppresses the development and progression of breast cancer (Mizushima et al., 2008). Understanding the role of autophagy during stress in the context of breast cancer cells will allow us to determine if autophagy could be a valuable drug target for breast cancer treatment. **Scope:** The goals of this research project are to: 1) Determine if breast epithelial cells (MCF10A cells) expressing oncogenes mutated in breast cancer initiate autophagy during extracellular matrix detachment, 2) Determine if autophagy inhibition promotes cell death during extracellular matrix detachment, alters 3D morphogenesis, and contributes to oncogenic transformation, and 3) Determine if autophagy suppression increases cell death and alters transformation in established breast cancer cell lines. Over the entire funding period of this award we have found that autophagy is induced in oncogene expressing breast epithelial cells and in two breast cancer cells lines in response to extracellular matrix detachment. Importantly we have found that autophagy is critical for the fitness of these cells as autophagy inhibition decreases the transformation potential of oncogene expressing breast epithelial cells and a breast cancer cell line, MDA-MB-231. Finally, we have found that autophagy inhibition alters 3D morphogenesis of breast epithelial cells expressing constitutively active PI3K and H-Ras.

BODY: Below is a summary of results from all tasks outlined in the approved Statement of Work that have been completed to date.

Task 1: *Determine if breast epithelial cells expressing activated PI3K and HER2/NEU induce autophagy during ECM detachment.*

Detailed results from Task 1 are described in full in the annual report from year one. In brief, we have found that autophagy is robustly induced in MCF10A cells expressing H-RasV12, PI3KH1047R and NeuT following extracellular matrix detachment. By western blot, we observed an increase in LC3-II levels in each of these oncogene expressing lines following matrix detachment that were further increased by the addition of lysosomal inhibitors (Tasks 1A and 1C). These results indicate that autophagy is induced following extracellular matrix detachment in these oncogene-expressing lines and that the autophagosomes that are formed during matrix detachment are being properly degraded in the lysosome. These results were confirmed using the above-described cells expressing GFP-LC3. Using GFP-LC3 expressing cells we observed an increase in GFP-LC3 "puncta" following extracellular matrix detachment that was comparable to levels observed in wild-type (non-oncogene expressing) MCF10A cells (Task 1B). Finally, we also observed a robust induction and turnover of autophagosomes in oncogene expressing MCF10A cells co-expressing mCherry-GFP-LC3 (Task 1D and 1E). Together these results indicate autophagy is robustly induced in MCF10A cells expressing

PI3KH1047R, NeuT or H-RasV12 following extracellular matrix detachment to levels comparable to wild-type MCF10A cells.

Task 2: *Determine if autophagy inhibition promotes the death of oncogene expressing cells during ECM detachment in both 2D and 3D models, and if autophagy contributes to oncogenic transformation.*

As described in detail in our annual reports from year one and year two we have found that genetic deletion of autophagy genes is sufficient to reduce oncogenic transformation. Briefly, we found that both H-RasV12 *atg5-/-, atg7-/-* and *atg3-/-*MEFs displayed a significant reduction in soft agar growth as compared to H-RasV12 wild-type controls and we observed a reduction in soft agar growth in PI3KH1047R *atg5-/-* MEFs compared to controls (Task 2G). Additionally, we found that apoptosis is enhanced in H-RasV12 expressing *atg5-/-* MEFs following extracellular matrix detachment compared to autophagy competent controls (Task 2C and 2D). Additionally, we generated MCF10A cell lines expressing an shRNA against ATG7. Expression of shATG7 in NeuT, H-RasV12 and PI3KH1047R MCF10A cells was sufficient to significantly reduce ATG7 protein levels and decrease autophagy in response to matrix detachment (Task 2B). Furthermore we cells found that autophagy inhibition was sufficient to reduce the transformation potential of H-RasV12 MCF10A cells in accordance with our results using H-RasV12 expressing MEFs (Task 2H).

We were able to successfully establish 3D cultures of NeuT, PI3KH1047R, and H-RasV12 MCF10A cells and compared the growth of shCNT expressing structures to structures expressing shATG7. Depending on the oncogene present, autophagy knockdown resulted in unique alterations in 3D morphogenesis (Task 2E). Knockdown of ATG7 in NeuT structures reduced the size of the structures but did not have an effect on structure morphology. In PI3KH1047R cells, knockdown of ATG7 did not alter structure size but did cause the clearance of luminal cells. Additionally in these structures we observed an increase in cleaved caspase-3 indicating an increase in luminal apoptosis following ATG7 knockdown (Task 2F). In contrast to the effects of autophagy knockdown on NeuT and PI3KH1047R structures, knockdown of ATG7 in H-RasV12 cells drastically altered structure morphology. H-RasV12 expression results in structures that are highly invasive, and autophagy knockdown was sufficient to reduce the invasive capacity of these structures. Following autophagy knockdown we also observed a partial restoration in cell-cell junction integrity and an increase in the levels of E-cadherin (Task 2F).

2E: *Set-up 3D morphogenesis assay in oncogene expressing MCF10A cell lines.*

Additional Findings:

As we reported in our previous annual reports and briefly describe above, inhibition of autophagy in H-RasV12 MCF10A cells results the suppression of invasive capacity during 3D morphogenesis. Additionally, we found autophagy inhibition is sufficient to partially restore cell-cell junction integrity and polarized secretion of the basement membrane protein laminin 5. In our year two annual report we showed that knockdown of ATG7 was also sufficient to rescue the epithelial marker E-cadherin in monolayer growth conditions. We have followed up on this finding and have compared the protein levels of other epithelial and mesenchymal markers, known to contribute to cell invasion and migration, between MCF10A H-RasV12 shCNT and shATG7 expressing cells that we have collected from 3D culture. Strikingly, we have found that

in addition to a restoration in E-cadherin we also observe a reversal in the expression levels of several epithelial and mesenchymal markers (Figure 1). We observed an increase in the epithelial marker keratin 14 (which is decreased following H-RasV12 expression) and a decrease in the mesenchymal markers fibronectin and vimentin (which are enhanced following H-RasV12 expression). These results indicate that in addition to supporting invasion during 3D morphogenesis autophagy also supports the mesenchymal differentiation that occurs following constitutive Ras activation. Since increased invasive capacity and changes in differentiation to a more mesenchymal state are thought to contribute to enhance breast cancer cell metastasis, these results suggest autophagy may be important in facilitating the later stages of breast cancer.

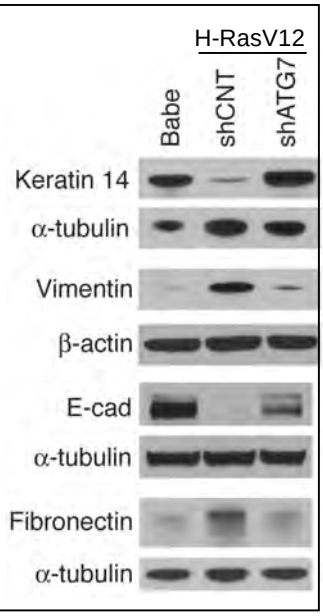

Figure 1: Western blot of epithelial markers (keratin 14 and E -cadherin) and mesenchymal markers (vimentin and fibronectin) in non-Ras (Babe) and H-RasV12 MCF10A cells expressing shCNT or shATG7 collected from 3D cultures on day 8. Knockdown of ATG7 in H-RasV12 cells results in the restoration of E-cadherin and keratin 14 and in a decrease in vimentin and fibronectin.

2F: *Measure apoptosis (cleaved-cleaved caspase 3) in 3D structures:*

As described in our year 2 annual report when examining MCF10A PI3KH1047R structures expressing shCNT or shATG7 we observed darker depressions in the centers of shATG7 expressing structures. Additionally, we observed increased levels of cleaved caspase-3 in the center of shATG7 expressing structures. We have now repeated this result several times and have quantified the level of luminal apoptosis in shATG7 expressing structures compared to shCNT and have found over a 20% increase in the number of apoptotic cells following autophagy knockdown (Figure 2).

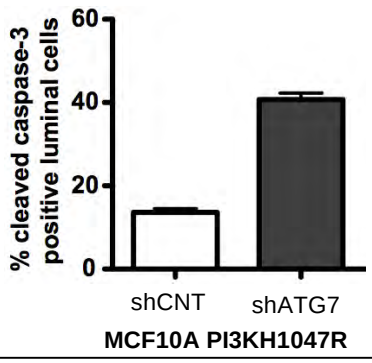

Figure 2: Percent of cleaved caspase-3 positive luminal cells counted in MCF10A PI3KH1047R shCNT or shATG7 expressing structures on day 7.

2H: *Assess transformation potential of MCF10A stable autophagy knockdown lines by quantifying colony formation in soft agar.*

In our year 2 annual report we described difficulties in growing MCF10A PI3KH1047R cells in soft agar to assess the contribution of autophagy towards the transformation potential of activated PI3K in breast epithelial cells. We decided to regenerate this cell line and have found that these newly generated PI3KH1047R expressing MCF10A cells are able to form colonies in soft agar. Therefore, we took these cells and stably expressed shATG7 to inhibit autophagy. We plated these cells along with shCNT expressing cells in soft agar and evaluated the ability of the cells to form colonies. Similar to what we observed following knockdown of ATG7 in H-

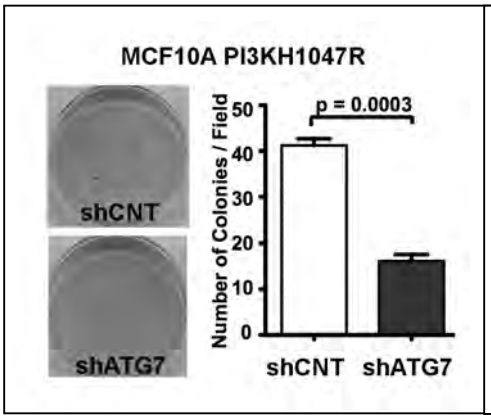

Figure 3: Images of soft agar colonies (left) and quantification of the number of colonies per field (right) formed by MCF10A PI3KH1047R cells expressing shCNT or shATG7.

RasV12 expressing MCF10As we observed a drastic reduction in the number of colonies that were formed following knockdown of ATG7. Following quantification of the number of colonies in each condition from several experiments we observed over a 50% decrease in colony formation following autophagy knockdown that was statistically significant (Figure 3). This indicates that autophagy is critical for the transforming ability of constitutively active PI3K in breast epithelial cells.

Task 3: *Determine if autophagy suppression increases cell death in established breast cancer cell lines following matrix detachment and during anchorage independent transformation.*

3A: *Examine levels of autophagy by western blot of LC3-I and LC3-II in breast cancer cell lines following suspension.*

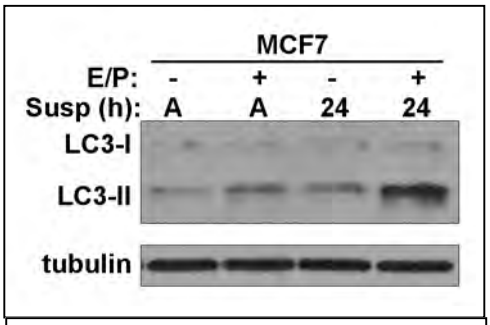

Figure 4: Levels of LC3-I and LC3-II MCF7 breast cancer cells following 24h of matrix detachment. MCF7 cells were plated either in attached (A) or suspension conditions for 24h (24). Lysosomal inhibitors E64d and pepstatin A (E/P) were added 5h before lysis to assess autophagic flux.

As described in our annual report from year 2 we found that autophagy is robustly induced in the breast cancer cell line MDA-MB-231 following matrix detachment. This was observed both by western blot (Task 3A) and by formation of GFP-LC3 "puncta" in MDA-MB-231 cells stably expressing GFP-LC3 (Task 3B). We have begun to evaluate whether this increase in autophagy occurs in other breast cancer cell lines by examining the levels of autophagy in MCF7 cells following extracellular matrix detachment. Similar to MDA-MB-231 cells, the levels of LC3-II increase in MCF7 cells following 24 hours of matrix detachment. Furthermore the addition of the lysosomal inhibitors E64d and pepstatin A to the culture further enhanced LC3-II levels indicating proper turnover of autophagosomes in the lysosome (Figure 4).

3C: *Stably express short-hairpin RNAs targeting ATG5 and ATG7 in breast cancer cell lines.*

We have started this task by stably expressing shRNAs against ATGs in MDA-MB-231 cells. Similar to what we describe in our year 1 report in MCF10A cell lines, we again achieved the highest level of knockdown with two unique shRNAs directed against ATG7 (shATG7-1 or shATG7-2) in MDA-MB-231 cells. Expression of these hairpins was sufficient to reduce ATG7 levels. Additionally, we found that expression

of shATG7 suppressed the induction of autophagy following 24 hours of extracellular matrix detachment by approximately 50% when compared to shCNT expressing cells (Figure 5).

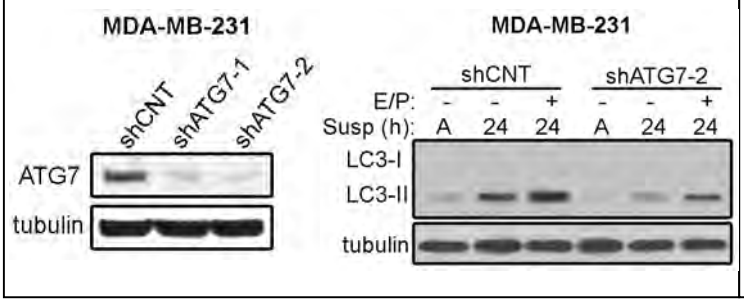

Figure 5: Levels of ATG7 (**left**) and LC3 (**right**) in MDA-MB-231 cells following knockdown of ATG7. **Right:** LC3-II levels in cells in attached conditions (A) or following 24h matrix detachment (24) in the presence or absence of E64d and pepstatin A (E/P).

3F: *Assess transformation potential of breast cancer cell lines expressing shATG7 verses shCNT by quantifying colony formation in soft agar.*

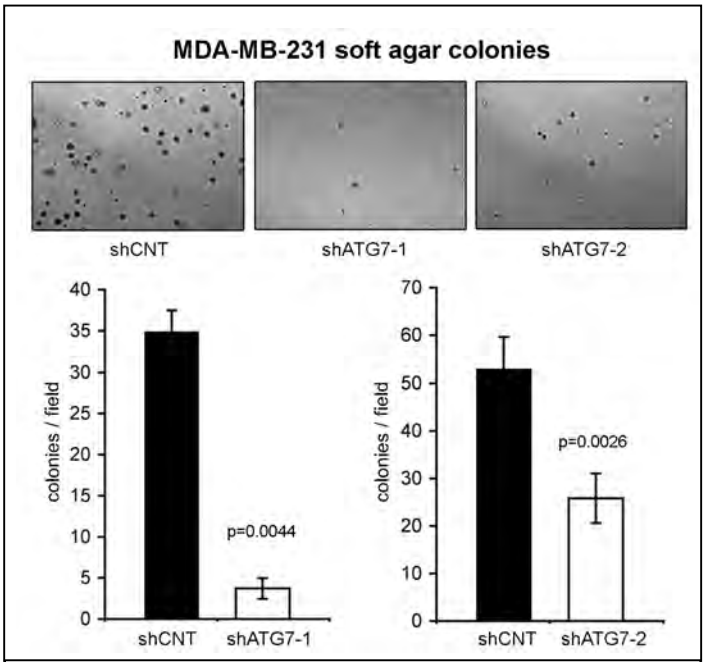

Figure 6: Representative images of soft agar colonies (top) and quantification of the number of colonies per field (bottom) from at least 3 independent experiments using MDA-MB-231 cells expressing shCNT or two independent shRNAs against ATG7 (ATG7-1 or ATG7-2).

Since we were able to generate lines of MDA-MB-231 cells stably expressing two unique shRNAs against ATG7, we were used these cells to evaluate if autophagy was critical to support the transformation potential of this breast cancer cell line. After 3 weeks of growth in soft agar, MDA-MB-231 cells formed small colonies that were visible under low magnification. In contrast, in the case of MDA-MB-231 cells expressing shATG7 (shATG7-1 or shATG7-2) the number of colonies that were formed in soft agar was significantly reduced. Following quantification, we found an approximate 7-fold and 2-fold reduction in colony formation following expression of shATG7-1 and shATG7-2 respectively (Figure 6). These results indicate that similar to what we observed in autophagy deficient MEFs and oncogene expressing MCF10A cells expressing shATGs, knockdown of ATG7 in MDA-MB-231 restricts growth in soft agar. This suggests a general requirement for autophagy in supporting anchorage independent growth.

3F Additional Findings:

Following knockdown and repeated passaging of MDA-MB-231 cells we noticed a potential decrease in the proliferation capacity of cells expressing shATG7. We examined this

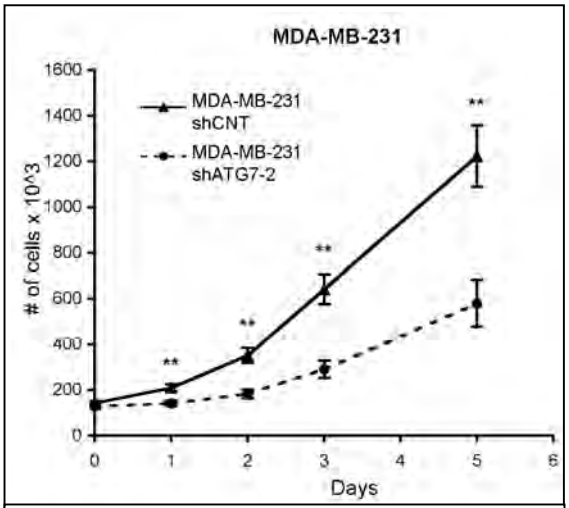

Figure 7: Five day growth curves of MDA-MB-231 cells expressing shCNT (solid line) or shATG7 (dashed line). ** Represents $p<0.01$

more carefully by comparing the growth curves of MDA-MB-231 cells expressing a control shRNA (shCNT) to those expressing shATG7 over a five-day period. As hypothesized from our initial observations, knockdown of ATG7 in MDA-MB-231 cells resulted in a significant decrease in the proliferation rate (Figure 7). We speculate that the decrease in proliferation that occurs following knockdown of ATG7 in MDA-MB-231 cells is a major contributor to the decrease in soft agar colony formation that occurs following autophagy knockdown.

KEY RESEARCH ACCOMPLISHMENTS:

Year 1:

- Western blot analysis of autophagy induction following matrix detachment of oncogene expressing MCF10A cells
- Confirmation of autophagy induction in oncogene expressing MCF10A cells by examination of both GFP-LC3 puncta and mCherry-GFP-LC3 puncta following matrix detachment.
- Testing and verifying short hairpins against ATGs in MCF10A cell lines
- Characterization of 3D morphogenesis phenotype resulting from autophagy inhibition in H-RasV12 expressing MCF10A cells
- Evaluation of apoptosis levels during 3D morphogenesis in RasV12 MCF10A cells expressing shATG7

Year 2:

- Generation of PI3KH1047R and NeuNT MCF10A cells expressing short-hairpins against critical autophagy genes.
- Characterization of 3D morphogenesis phenotypes resulting from autophagy inhibition in PI3KH1047R and NeuNT expressing MCF10A cells.
- Evaluation of cleaved-caspase 3 levels (apoptosis levels) in PI3KH1047R MCF10A cells expressing shATG7 during 3D morphogenesis.
- Western blot analysis of autophagy induction in MDA-MB-231 cells following 24h extracellular matrix detachment.
- Generation of MDA-MB-231 cells expressing GFP-LC3. Confirmation of autophagy induction following 24h suspension in MDA-MB-231 cells expressing GFP-LC3.

Year 3:

- Characterization of epithelial and mesenchymal differentiation markers in non-Ras expressing (Babe), H-RasV12 shCNT, and H-RasV12 shATG7 MCF10A cells collected from 3D culture.
- Quantification of luminal cell death (cleaved caspase-3 staining) in MCF10A PI3KH1047R shCNT and shATG7 expressing 3D structures.
- Assessed transformation potential (growth in soft agar) of MCF10A PI3KH1047R cells expressing shCNT or shATG7
- Western blot analysis of autophagy induction in MCF7 cells following 24h extracellular matrix detachment.
- Stable expression of shRNAs against shATG7 in MDA-MB-231 cells
- Assessed transformation (growth in soft agar) and proliferation of MDA-MB-231 cells expressing shCNT or shATG7.

REPORTABLE OUTCOMES:

Publications:

Lock R, Kenific C, and Debnath J. Autophagy promotes cell motility and invasion driven by oncogenic Ras. Manuscript in preparation.

Lock R, Debnath J. Ras, autophagy and glycolysis. *Cell Cycle* 2011 May 15;10(10).

Lock R, Roy S, Kenific CM, Su JS, Salas E, Ronen SM, Debnath J. Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. *Mol Biol Cell* 2011, 2:165-78

Lock R, Debnath J. Extracellular matrix regulation of autophagy. *Curr Opin Cell Biol*. 2008, 5:583-8

Abstracts:

1. Autophagy inhibition alters glucose metabolism and promotes epithelial differentiation during Ras-mediated oncogenic transformation. Rebecca Lock, Candia Kenific, Srirupa Roy, Eduardo Salas, and Jayanta Debnath. Keystone Symposia: Cell Death Pathways Apoptosis, Autophagy and Necrosis / Metabolism and Cancer Progression. March 12-17, 2010.

- Short talk presenter
- Poster presenter

2. Autophagy inhibition alters glucose metabolism and promotes epithelial differentiation during Ras-mediated oncogenic transformation. Rebecca Lock, Candia Kenific, Srirupa Roy, Eduardo Salas, and Jayanta Debnath. Gordon Conference on Autophagy in Stress, Development and Disease. April 25-30, 2010.

- Poster presenter

3. Autophagy supports cell motility and invasion driven by oncogenic Ras. Rebecca Lock, Candia Kenific, and Jayanta Debnath. Molecular Therapeutics of Cancer Research Conference. July 10-14 2011.

- Poster presenter

4. Autophagy inhibition in breast cancer cells decreases anchorage-independent growth and alters 3-D morphogenesis. Rebecca Lock, Nan Chen, Candia Kenific, and Jayanta Debnath. BCRP Era of Hope Conference. August 2-5 2011.

- Poster contest winner

CONCLUSION:

Since the funding of this award, we have determined that autophagy is induced in breast epithelial cells (MCF10A) expressing oncogenes that activate the PI3K and Ras pathways both by western blot analysis of LC3-II levels and by visualization of GFP-LC3 and mCherry-GFP-LC3 puncta formation. Additionally, we have observed robust autophagy induction in MDA-MB-231 and MCF7 cells, two breast cancer cell lines. This result is important in understanding the role of autophagy in early stages of breast cancer development as cells begin to grow independent of proper extracellular matrix contact.

Next, we have validated short-hairpin RNAs against autophagy genes that are able to not only achieve knockdown of the intended targets, but also significantly inhibit autophagy induction in both oncogene expressing MCF10A cells as well as in MDA-MB-231 cells. These shRNAs were critical in evaluating the contribution of autophagy during 3D morphogenesis and transformation. Following the generation of these lines, we have found that H-RasV12 and PI3KH1047R MCF01A cells as well as MDA-MB-231 cells expressing shATG7 display a significant reduction in soft agar growth compared to controls. These results indicate a critical function for autophagy in supporting the anchorage independent growth of breast cancer cells.

Finally, we have extensively evaluated the role of autophagy during 3D morphogenesis of oncogene expressing breast epithelial cells using H-RasV12 MCF10A and PI3KH1047R MCF10A cells expressing shATG7. Autophagy inhibition in H-RasV12 3D structures decreased both the growth and invasive capacity of these structures. The decrease in invasive capacity following autophagy reduction correlated with a restoration in normal cell-cell junctions, polarized secretion of the basement membrane protein laminin 5, and a restoration in epithelial differentiation markers and a decrease in mesenchymal differentiation markers. In contrast, we have found that PI3KH1047R MCF10A cells expressing shATG7 form 3D structures that are grossly indistinguishable from shCNT structures in both size and shape. However, upon closer characterization we found that knockdown of ATG7 in PI3KH1047R structures was sufficient to cause luminal cell clearance associated with a significant increase in luminal cell apoptosis. These results suggest the function of autophagy is highly context dependant and might vary depending on the oncogenic insult present. In the case of H-RasV12 3D morphogenesis our preliminary results suggest a potential role for autophagy in supporting invasion and metastasis during later stages of breast cancer development. In contrast, inhibition of autophagy in the presence of constitutively active PI3K, results in decreased survival of luminal cells deprived of basement membrane contact suggesting a critical role for autophagy in supporting the survival of breast cancer cells at the earliest stages of breast cancer development. Overall, our results have

contributed towards the understanding of how autophagy might be modulated during breast cancer development and how it might enhance breast cancer progression.

REFERENCES:

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D. (2008). Autophagy fights disease through cellular self-digestion. Nature. *451*, 1069-1075.

APPENDICES:

None.

SUPPORTING DATA:

Embedded in text.